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Enhanced production of recombinant rabies virus glycoprotein (rRVGP) by *Drosophila melanogaster* S2 cells through control of culture conditions

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Abstract Culture conditions that affect product quality are important to the successful operation and optimization of recombinant protein production. The objective of this study was to optimize culture conditions for growth of recombinant *Drosophila melanogaster* S2 cells (S2AcRVGP) in order to enhance the production of rRVGP. The addition of DMSO and glycerol to the medium and growth at a reduced temperature (22 °C) were the culture condition variations selected to be tested. Experimental cultures were first performed in serum-free Sf900 II medium in 250 ml Schott flasks. The most promising conditions identified in these experiments were also tested on a higher scale in a 3l bioreactor. In the Schott flasks experiments, all the changes in culture conditions resulted in an increase of rRVGP production. The protein concentration was 3.6-fold higher with addition of 1% DMSO and 1% glycerol and 9.3-fold higher when the cells were cultured at 22 °C instead of the standard 28 °C. The maximum concentration of rRVGP reached was 591 μg l^{-1}. In bioreactor experiments, with control of pH at 6.20 and DO at 50%, the reduced culture temperature (22 °C) was the strategy that promoted the highest glycoprotein production, 928 μg l^{-1}.

Keywords *Drosophila melanogaster* S2 cells · Dimethyl sulfoxide · Glycerol · Rabies virus glycoprotein · Recombinant protein production · Reduced temperature cultivation

Abbreviations

rRVGP	Recombinant rabies virus glycoprotein
μ_{max}	Maximum specific growth rate (h^{-1})
X_{max}	Maximum cell density (cell ml^{-1})
P^{X}_{max}	Maximum cell productivity (10^5cell ml^{-1} h^{-1})
C^{rRVGP}_{max}	Maximum rRVGP concentration (μg l^{-1})
$\bar{q}_{rRVGP}$	Specific production rate of rRVGP (μg 10^{-6}cell h^{-1})
P^{rRVGP}_{max}	Maximum rRVGP productivity (μg l^{-1} h^{-1})

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Introduction

Many culture strategies are being used to improve the productivity of insect cell bioprocesses for recombinant protein production (Li et al. 2006a). One such strategy, the addition of supplements to the medium is effective, convenient and quick. Dimethyl sulfoxide

(DMSO) and glycerol have been used as additives in cell culture systems. Both compounds have been shown to act as chaperones, stabilizing proteins during protein folding (Yoshida et al. 2002). Fiore and Degrassi (1999) reported that DMSO caused CHO cell growth arrest, prevented apoptosis, and promoted protein production. Glycerol has been used extensively in the pharmaceutical and biotechnological industry as a protein stabilizer. It can form hydrogen bonds and aid the formation of a solvent shell around protein molecules (Fagain 1997).

In recent years there have been a growing number of reports on the use of low temperature culture (cold-shock) of mammalian cells for the enhanced production of recombinant proteins (Al-Fageeh et al. 2006). Among other beneficial physiological effects, this technique results in prolonged generation time, maintenance of high cell viability and reduced protease activity (Yoon et al. 2003).

The rRVGP has been produced in several expression systems: [transgenic plants (Ashraf et al. 2005), yeast cells (Sakamoto et al. 1999), mammalian cells (Kankanamage et al. 2003), and insect Sf9 cells infected with baculovirus (Drings et al. 1999)]. However, these reports have focused on the immunogenicity and antigenicity characteristics and also on the conformational structure of the protein, providing little satisfactory information about expression levels, which is important for developing a bioprocess for production of large quantities of protein.

In this study we have investigated the effects of culture conditions on rRVGP production and S2AcRVGP cell growth in small scale flasks as well as in a bioreactor. The immunogenicity and antigenicity of the rRVGP produced in S2 cells were characterized by Yokomizo et al. (2007), who demonstrated that the protein is immunogenic and induces protection against experimental rabies virus infection. The data obtained by these authors encouraged this quantitative study of rRVGP expression in S2 cells for production of a biomolecule that can be biologically characterized and used as an efficient rabies vaccine. Besides, no report has been published on the improvement of recombinant protein production with the *Drosophila* expression system. Three strategies were tested with the aim of enhancing rRVGP production: the addition of DMSO and glycerol at levels around 1% v/v and culturing the cells at reduced temperature (22 °C). These methods were selected, since they have been reported to improve glycoprotein production in mammalian cells (Yoon et al. 2003; Fox et al. 2004; Rodriguez et al. 2005).

Materials and methods

Cells and culture conditions

Recombinant S2 cells transfected by Yokomizo et al. (2007) (S2AcRVGP) for rRVGP production and stored in liquid nitrogen at −196 °C were used. The cells were cultured in the serum-free medium Sf900 II (Gibco BRL) in 250 ml Schott flasks and a 3l bioreactor. Experiments on the small scale were conducted in Schott flasks containing 30 ml medium with an initial cell seeding of 5×10^5 cells ml^{-1}, at 100 rpm and 28 °C.

Large-scale cultures were carried out in a 3l Bioflo 110 bioreactor (New Brunswick Scientific, NJ, USA) with 1 l of working volume, equipped with a pitched-blade impeller, pH control at 6.20 and dissolved oxygen (DO) control at 50% air saturation, oxygenation being carried out by diffusion through a silicon membrane and increasing the stirring speed (15 rpm day^{-1}), starting at 100 rpm, up to a maximum of 250 rpm. The experiments were done at 28 °C with an initial cell seeding of 5×10^5 cell ml^{-1}, the cells being transferred from a mid-exponential 500 ml spinner flask (Wheaton®) culture. Exceptionally, an experiment carried out at 22 °C was inoculated with 10^6cell ml^{-1}.

Analytical methods

The total cell density was determined with a hemacytometer and the viability by the trypan blue exclusion method (Doyle and Griffths 1998). The rRVGP is a transmembrane protein present in cell lysates which were evaluated by ELISA (enzyme-linked immunosorbent assay) with immunoglobulin (IgG) against rabies glycoprotein (RVGP), purified from the serum of rabbits immunized with purified RVGP from rabies virus (Pasteur lineage) propagated on Vero cells, as a cover antibody. The cover antibody labeled with peroxidase was used to detect bound rRVGP antigen as described by Astray et al. (2008). The accuracy of the assay is set to 10 μg l^{-1}. The kinetic parameters were evaluated in agreement with Doyle and Griffths (1998).

Results and discussion

In an attempt to increase recombinant rRVGP production by an insect cell line, some culture strategies were tested about which little has been published. Firstly, the effect of supplementing the medium with glycerol and DMSO and, secondly, the effect of temperature, were evaluated in Schott flask cultures. The most promising conditions identified in Schott flasks experiments were also tested on a higher scale in a stirred tank 3l bioreactor.

Cultures in Schott flasks

Figure 1 shows the cell performance and rRVGP production in Schott flasks experiments and Table 1 the kinetic parameters for the culture strategies tested.

- Effect of glycerol. The addition of 1% glycerol practically did not modify cell growth. The specific growth rate (μ_{max}) and maximum cell density (X_{max}) were similar to control cultures. However, the rRVGP concentration (C_{max}^{RVGP}) was 3.6-fold higher than in the control culture, reaching 232 μg l^{-1}. The specific production ($\bar{q}_{RVGP}$) rate and maximum productivity (P_{max}^{RVGP}) were also higher, 5.3 and 4.6-fold, respectively. However, a high drop in protein concentration was observed in the experiment with added glycerol. The rRVGP concentration started to fall after 4 days of culture. Analysis of amino acids (results not shown) revealed the exhaustion of cysteine by that time. As this amino acid is well known for its role in the formation of covalent sulfur bridges that are of crucial importance in protein stabilization (Freedman 1984), its lack could be the cause of the intense degradation of the glycoprotein.
- Effect of DMSO. In the Schott culture supplemented with 1% DMSO, μ_{max} and X_{max} were 18 and 10% lower, respectively, than in the control culture. Although the specific production rate of rRVGP in the presence of 1% DMSO was not enhanced as reported in the literature on recombinant protein production by mammalian cells (Rodriguez et al. 2005; Li et al. 2006a, b), the volumetric concentration and maximum productivity were 3.6 and 1.9-fold higher, respectively. Also, no drop of rRVGP concentration was observed in the experiment with added DMSO.
- Effect of temperature. Despite the fact that culture at reduced temperature has been widely used in the production of recombinant products from mammalian cells (Al-Fageeh et al. 2006), this strategy has not been employed yet to enhance protein production in insect cells. The culture of S2AcRVGP cells at reduced temperature (22 °C) resulted in significant reduction in cell growth. The μ_{max} value was approximately twofold lower in the culture at 22 °C than in the control culture at 28 °C and X_{max} was considerably reduced by about 22%. As a result of the reduction in cell growth, there was a large increase in rRVGP production. The rRVGP concentration increased about 10 times, $\bar{q}_{RVGP}$ and P_{max}^{RVGP} about 5.3 and 4

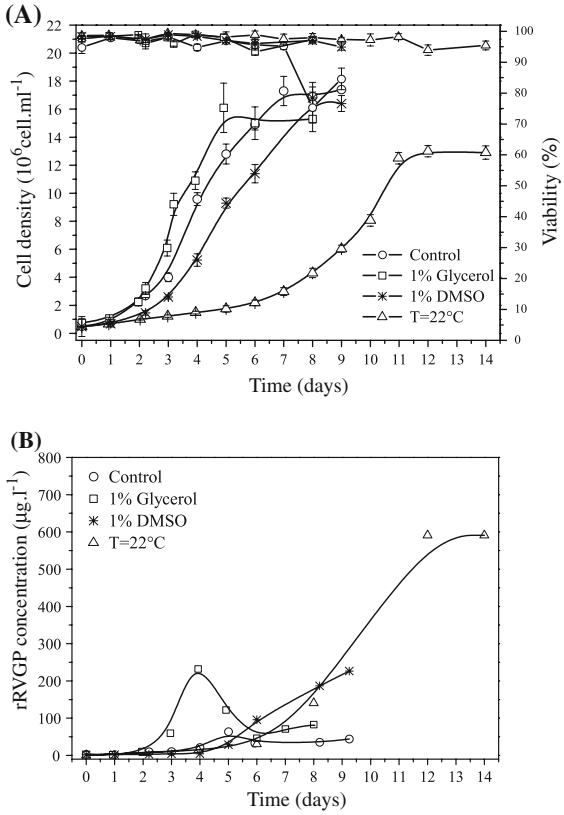

Fig. 1 (**a**) Growth of S2AcRVGP cells and (**b**) rRVGP production in 250 ml Schott culture experiments in shaker at 100 rpm. In the control culture and in the experiment at 22 °C just Sf900 II medium was utilized. In the other two experiments supplements were added in the concentrations indicated. The *bars* represent the SE

Table 1 Kinetic parameters for S2AcRVGP cell growth and rRVGP production in Schott flask at 100 rpm in Sf900 II medium

	Growth[a]			rRVGP production[a]		
	μ_{max} (h^{-1})	X_{max} (10^6cell ml^{-1})	P^X_{max} (10^5cell ml^{-1} h^{-1})	C^{rRVGP}_{max} (μg l^{-1})	$\bar{q}_{rRVGP}$ (μg 10^{-6}cell h^{-1})	P^{rRVGP}_{max} (μg l^{-1} h^{-1})
Control	0.034 ± 0.003	17.8	1.05	63.8	0.04	0.53
Glycerol 1%	0.039 ± 0.003	16.1	1.37	232	0.21	2.45
DMSO 1%	0.028 ± 0.001	16.8	0.88	227	0.05	1.02
$T = 22$°C	0.015 ± 0.001	13.9	0.45	591	0.21	2.05

[a] P^{rRVGP}_{max} and $\bar{q}_{rRVGP}$ were calculated only in the increasing rRVGP concentration region

times, respectively. The reduction in the volumetric concentration of recombinant protein, frequently reported in literature as a result of the reduced cell growth (Al-Fageeh et al. 2006), was not observed in the present study.

Analyzing the results of the Schott flasks experiments, it could be seen that the addition of 1% glycerol and performing the culture at reduced temperature were the strategies that gave the most satisfactory increases in the rRVGP production. However, the addition of glycerol was not efficient at preventing protein degradation.

The DMSO supplementation resulted in an increase in the volumetric concentration and productivity and, most importantly, the rRVGP degradation was not observed. The strategy that promoted the highest rRVGP production was the low temperature culture, which resulted in a low cell growth-rate and a considerable increase in protein production parameters. The rRVGP concentration was almost 10 times higher in the culture grown at 22 °C than in the culture at standard culture temperature, 28 °C, resulting in a substantial improvement of the bioprocess for rRVGP production. Therefore, the addition of 1% DMSO and the culture at reduced temperature were the strategies chosen to be tested in the bioreactor, in a more controlled culture environment.

The rRVGP levels produced in Schott flasks in this work (64–590 μg l^{-1}) were similar to the produced by Yokomizo et al. (2007), using the same culture conditions, where the rRVGP expression in cell lysates and supernatants attaining concentrations of 300 μg l^{-1}. Galesi et al. (2007) evaluated the rRVGP production in serum-free media especially designed for *Drosophila* cell growth (TC-100 supplemented with glucose, glutamine, lipid emulsion, Pluronic F68, yeastolate and soy hidrolysate) and found expression levels near 10 μg l^{-1}.

Cultures in bioreactor

Figure 2 shows the profiles of cell growth and rRVGP production and Table 2 the kinetic parameters determined in bioreactor experiments.

- Effect of DMSO. The addition of 1% DMSO to the bioreactor with control of pH at 6.2 and DO = 50%, as in the Schott flasks, resulted in a reduction of μ_{max} from 0.049 to 0.036 h^{-1}; X_{max} was also lower (equal to 1.76×10^7 cell ml^{-1}), than in the control experiment, in which it reached 2.10×10^7 cell ml^{-1}, as can be observed in Fig. 2. Despite the reduction in cell growth, the DMSO addition did not result in an increase of rRVGP production, contrary to what was observed in the Schott experiments. In the bioreactor, this culture strategy only enhanced the specific rRVGP production. In a more controlled culture environment, provided by the bioreactor, the effect of DMSO on protein production was quite different than that obtained in Schott flasks without pH and DO control.
- Effect of temperature. When subjected to sub-physiological culture temperature, S2AcRVGP cell growth was suppressed, reducing the μ_{max}-value from 0.049 to 0.026 h^{-1}. To avoid the prolonged lag phase observed in the experiment carried out in Schott flasks at 22 °C, the inoculum cell density in the bioreactor experiment was enhanced from 5.0×10^5 to 10.0×10^5 cell ml^{-1}. With this high inoculum cell density, the cells at 22 °C started to grow exponentially without a lag period and reached a maximum viable density of 16.1×10^6 cell ml^{-1}. In the

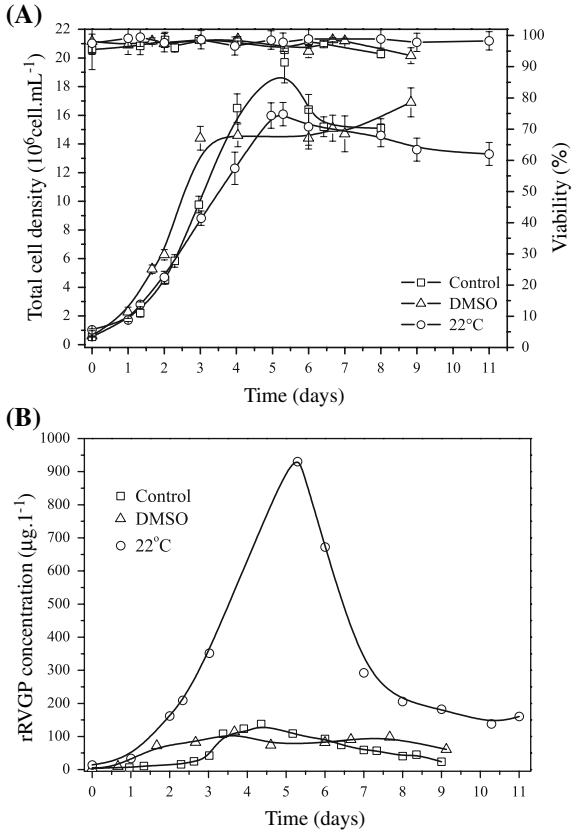

Fig. 2 (**a**) Growth of S2AcRVGP cells and (**b**) rRVGP production in bioreactor experiments with Sf900 II medium, pH = 6.2, DO = 50% and increasing stirring speed (15 rpm day^{-1}), starting at 100 rpm until a maximum of 250 rpm

control culture at 28 °C, with an inoculum density of 5.0×10^5cell ml^{-1}, the cells reached a maximum viable density of 20.0×10^6 cell ml^{-1}, also without a lag period. Focusing on trends rather than on absolute values, in view of the enhanced inoculum size used at 22 °C, it can be said that, as in the Schott flask experiment, the values of μ_{max} and $\bar{q}_{r\text{RVGP}}$ in the bioreactor showed similar behavior at both temperatures: reduced specific cell growth in favor of a marked rise in specific production of glycoprotein. The rapidly decreasing titer of rRVGP from 5 days may be a consequence of the depletion of cysteine and other amino acids, which should be due mainly to the high production of rRVGP, differently from what was observed in the case of addition of glycerol to Schott flasks cultures, in which the early exhaustion of amino acids was caused by high cell growth.

The notable differences observed in cell growth and rRVGP production between the cultures in Schott flasks and bioreactor can be explained by the better controlled conditions (pH and DO) attained in the bioreactor.

Conclusions

In this study, we explore three culture strategies in an attempt to enhance rRVGP production: addition of DMSO and glycerol and culture at a reduced temperature (22 °C). In small scale experiments (Schott flasks), the addition of DMSO and culture at 22 °C were the strategies that afforded the most satisfactory results. The protein expression was 3.6 and tenfold higher in the culture with DMSO and culture at 22 °C, respectively. There was also no degradation of rRVGP under those conditions. The culture of S2AcRVGP cells at 22 °C in the bioreactor gave rise to lower values of μ_{max} and to higher values of $\bar{q}_{r\text{RVGP}}$ than that at 28 °C. Even though the comparison of the strategies was not made at their optimal values, reducing the temperature can be

Table 2 Kinetic parameters determined in bioreactor experiments with S2AcRVGP cells for growth and rRVGP production

	Growth[a]			rRVGP Production[a]		
	μ_{max} (h^{-1})	X_{max} (10^6cell ml^{-1})	P^X_{max} (10^5cell ml^{-1} h^{-1})	$C^{r\text{RVGP}}_{max}$ (μg l^{-1})	$\bar{q}_{r\text{RVGP}}$ (μg 10^{-6}cell h^{-1})	$P^{r\text{RVGP}}_{max}$ (μg l^{-1} h^{-1})
Control	0.044 ± 0.001	20.0	1.90	138	0.12	1.32
DMSO	0.051 ± 0.003	17.6	0.95	114	0.32	1.30
22 °C	0.037 ± 0.003	16.1	1.26	928	0.73	7.20

[a] $P^{r\text{RVGP}}_{max}$ and $\bar{q}_{r\text{RVGP}}$ were calculated only in the increasing rRVGP concentration region

considered the most attractive. Similarly, compared to other strategies of growth regulation, low temperature cultivation has been proved to be the most reliable and efficient for CHO cells (Kumar et al. 2007). A more complete study of the effect of reduced temperature cultivation on S2AcRVGP cells, to find the optimal temperature for rRVGP is being conducted in our laboratory.

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References

Al-Fageeh MB, Marchant RJ, Carden MJ et al (2006) The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. Biotechnol Bioeng 93:829–835

Ashraf S, Singh PK, Yadav DK et al (2005) High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J Biotechnol 119:1–14

Astray RM, Augusto E, Yokomizo AY et al (2008) Analytical approach for the extraction of recombinant membrane viral glycoprotein from stably transfected Drosophila melanogaster cells. Biotech J 3:98–103

Doyle A, Griffths JB (1998) Cell and tissue culture: laboratory procedures in biotechnology. Hardcover, England

Drings A, Jallet C, Chambert B et al (1999) Is there an advantage to including the nucleoprotein in a rabies glycoprotein subunit vaccine? Vaccine 17:1549–1557

Fagain CO (1997) Stabilizing protein function. Springer, New York

Fiore M, Degrassi F (1999) Dimethyl sulfoxide restores contact inhibition-induced growth arrest and inhibits cell-dependent apoptosis in hamsters cells. Exp Cell Research 25:102–110

Fox SR, Patel UA, Yap MGS et al (2004) Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling. Biotechnol Bioeng 85:177–184

Freedman R (1984) Native disulphide bond formation in protein biosynthesis: evidence for the role of protein disulphide isomerase. Trends Biochem Sci 9:438–441

Galesi ALL, Pereira CA, Moraes AM (2007) Culture of transgenic *Drosophila melanogaster* Schneider 2 cells in serum-free media based on TC100 basal medium. Biotechnol J 2:1399–1407

Kankanamage PJ, Irie T, Shoji J et al (2003) Further characterization of the rabies virus glycoprotein produced by virus-infected and G cDNA-transfected cells using a monoclonal antibody, #1-30-44, which recognizes an acid-sensitive epitope. Microbiol Immunol 47:337–349

Kumar N, Gammell P, Clynes M (2007) Proliferation control strategies to improve productivity and survival during CHO based production culture. Cytotechnology 53:33–46

Li J, Huang Z, Sun X et al (2006a) Understanding the enhanced effect of dimethyl sulfoxide on hepatitis B surface antigen in the culture of Chinese hamster ovary cells on the basis of proteome analysis. Enzyme microbial technol 38:372–380

Li J, Sun X, Zhang Y (2006b) Improvement of hepatitis B surface antigen expression by dimethyl sulfoxide in the culture of recombinant Chinese hamster ovary cells. Proc Biochem 41:317–322

Rodriguez J, Spearman M, Huzel N et al (2005) Enhanced production of monomeric interferon-β by CHO cells through the control of culture conditions. Biotechnol Prog 21:22–30

Sakamoto S, Ide T, Tokiyoshi S et al (1999) Studies on the structures and antigenic properties of rabies virus glycoprotein analogues produced in yeast cells. Vaccine 17:205–218

Yokomizo AY, Jorge SAC, Astray RM et al (2007) Rabies virus glycoprotein expression in Drosophila S2 cells. I. Functional recombinant protein in stable co-transfected cell line. Biotechnol J 2:102–109

Yoon SK, Song JY, Lee GM (2003) Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng 82:289–298

Yoshida H, Yoshizawa T, Shibasaki F et al (2002) Chemical chaperones reduce aggregate formation and cell death caused by the truncated Machado Joseph disease gene product with an expanded polyglutamine stretch. Neurobiological Diseases 10:88–99